AN EXPERIMENTAL ANALYSIS OF FERTILIZATION IN PLATYNEREIS MEGALOPS.

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Study of the breeding habits of *Platynereis megalops* revealed the fact, as has been pointed out (Just, '14), that insemination takes place in the body cavity of the female and that although egg laying begins often but five seconds after copulation, the eggs will not fertilize when artificially inseminated after exposure to the action of sea-water. It is this failure of sea-water insemination that forms the basis of the present contribution to the analysis of fertilization in *Platynereis*. In order clearly to interpret the phenomena of sea-water insemination a study of the morphology of the normal fertilization was made (see Just, '15*a*).

The experiments undertaken for the analysis of fertilization in *Platynereis* come under three heads:

A. Conditions of successful insemination.

B. Cross fertilization with *Nereis*.

C. Artificial parthenogenesis with various agents.

B and C are taken up mainly because they supplement results under A.

A. Conditions of Successful Insemination.

During the summer of 1911, I was studying the maturation and fertilization of the *Platynereis* egg for comparison with those processes in *Nereis*. The methods of insemination used with *Nereis*, cutting out the eggs and sperm in sea-water, gave no cleavage. Various trials with the utmost care, using diverse methods never gave cleavage. Not until August 24, 1911, did I chance to find that normally insemination takes place in the body cavity of the female (cf. Just, '14).

1. *Observations on Eggs Inseminated in Sea-water.*

If eggs and sperm be cut out of *Platynereis* and mixed in sea-water, the phenomena of maturation, sperm attachment, and

copulation of the germ nuclei may be readily followed; but such eggs do not segment nor do they ever develop into swimming forms.

The Living Egg.

If insemination be made in a suspension of India ink ground up in sea-water, the jelly formation may be easily followed: it differs but little from the cortical outflow observed in eggs normally laid. All eggs, however, do not secrete this jelly; of these, some remain in the germinal vesicle stage and others go through maturation with all or part of the cortex intact.

As in the normally inseminated egg (see Just, '15*a*) no cone is present. More often than in the normally laid egg a broad plateau of cytoplasm marks the point of sperm attachment. The sperm, from one to six, are attached to the membrane above this raised cytoplasm or near it.

Maturation proceeds about as in the normal egg. At maturation stages slightly later than in the normal egg, the sperm may be found in the egg. It moves forward with aster formation. The pronuclei meet, remain apposed for a short time, separate, and fade from view. This is not true of all eggs; for apparently, those in the germinal vesicle stage or in maturation stages with cortex intact never engulf the sperm. Moreover, in many eggs that are in maturation with the cortical layer gone, one cannot find sperm.

These eggs never divide. At first, 1911, I thought that this behavior of the egg was due to injury of the worms. Its significance became clear only after the discovery of the normal method of egg-laying.

The Sectioned Egg.

During four seasons eggs have been preserved at three and five minute intervals upward to two hours after insemination in sea-water. Study of the sectioned eggs confirms the findings of the study of living eggs. Many eggs remain ovocytes with sperm attached or not. Those that go through maturation do so with or without jelly formation. Eggs that form jelly are likewise of two classes: those in which sperm are found to have penetrated and those in which no sperm are found.

I have not been able so far to determine any structural differences in the ovocytes with and without sperm attached. In the case of the eggs that maturate with the cortex wholly or partially intact, the spindle may be abnormal. In most cases if it reach the periphery of the egg it does so at a point practically devoid of cortical cytoplasm. Or again, it may lie parallel to a tangent of the egg membrane.

Those sections which reveal the sperm within the egg are in the minority. It appears from experiments several times repeated during the four seasons of study that the penetration of the sperm depends upon the amount of sea-water used. If the eggs be inseminated in a large quantity of sea-water or washed (by changing the water several times) very few eggs form jelly. With less water more form jelly. Eggs inseminated quickly in small quantities of sea-water are capable of engulfing sperm.

The history of the penetration as known may be briefly given. One finds sperm external to the egg at different stages. How it gets into the egg I cannot yet state with certainty although this point has received most careful study for three years. Material has been prepared in every way possible to demonstrate the early penetration. So far I have not found the sperm entering the egg as a slender thread like that in the normal egg. It can be easily demonstrated in the endoplasm. On one slide of the 1911 series, for instance, I counted twenty sperm heads with their asters lying near the centre of the egg. The sperm head remains for a longer time than in the normal egg a black knot with a long drawn out thread extending to the single aster. A second aster has never been found. The germ nuclei copulate but the eggs never cleave. Various stages are found from sixty to one hundred twenty minutes after insemination—sixty minutes after cleavage in the normal egg. The pronuclei after apposition gradually separate and degenerate as discrete nuclear masses. Many eggs show only one chromatin mass in process of degeneration; doubtless, these are eggs which sperm do not enter. The sections of such eggs closely resemble those of *Nereis* eggs from which the sperm have been removed (see Lillie, '12). I have repeatedly made observations on living eggs inseminated in sea-water and on sections. I have yet to find a single cleaving egg.

Two hours after insemination the eggs exhibit cytoplasmic stratification; the oil drops later fuse to form one at the vegetative pole. Twelve hours after insemination the conditions are the same; there is never a swimming form among these eggs.

2. *Nature of the Inhibition to Development.*

It may be very clearly shown that sea-water is responsible for the lack of cleavage by the method of "dry insemination." If males and females dried on filter paper be cut up separately and the drops of eggs and sperm thus obtained be mixed with subsequent addition of sea-water, a percentage of the eggs always cleave and develop into normal trochophores. I have kept larvae from such dry inseminations until they were seven mm. long with thirty or more segments, few differing from normally laid eggs. There is doubtless an optimum time after mixing for the addition of sea-water, but any time upward to two minutes gives results. The following is an example:

August 3, 1912. To determine the time interval after mixing dry eggs and sperm before adding sea-water.

Water Added.	Per Cent. of Cleavage.
1. At once	60
2. Five seconds after	50
3. Ten seconds after	90
4. Twenty seconds after	45

Practically, as soon as eggs and sperm are mixed, sea-water may be added. I have not been able to add sea-water quickly enough after mixing to prohibit cleavage. If the eggs are allowed to stand two minutes the majority are plasmolyzed by the addition of sea-water.

The amount of sea-water that will permit fertilization has been repeatedly determined:

July 28, 1912, 9:45 P.M. Experiment to determine the maximum amount of sea-water that permits fertilization.

Males and females are thoroughly dried on clean filter paper. A male and a female placed in each of the eight perfectly dried clean watch glasses. Sea-water added as follows:

No. 1	1 drop.
" 2	2 drops.
" 3	3 "
" 4	4 "
" 5	5 "
" 6	6 "
" 7	10 c.c.
" 8	no sea-water.

The worms were then cut up and flooded with sea-water, later transferred to fresh sea-water in finger bowls.

Nos. 1, 2, 3 and 8 gave cleavage; a per cent. of normal trochophores was found the next morning. In dishes 4, 5, 6 and 7 not an egg divided, no swimming forms developed.

No single observation in the whole work was made as often as this; the results are wonderfully precise. As I shall show later the experiment quoted was conducted under the optimum conditions, and yet it shows the inhibiting effect of such a surprisingly small quantity of sea-water. All other observations show two drops of sea-water for each worm to be the maximum that will permit normal fertilization. In no case have I got cleavage where two and one-half drops of sea-water for each worm (*i. e.*, five drops to two worms) were used. While the same pipette was used to secure equal drops, the worms, females particularly, vary in size. I have usually taken the average females for these experiments. Such an animal, as found by actual count in three cases, has about 11,000 eggs. There is enough variation, however, in the size and weight of the worms to make impossible any law concerning the lethal amount of sea-water. I believe, nevertheless, that there is an optimum time for the addition of sea-water—equal to the time the sperm are in the female in normal insemination; and an optimum amount of sea-water—about as much as the worms will take up after thorough drying.

The results of these inseminations over a period of four seasons prove clearly that sea-water except in minute quantity is fatal to fertilization.

Does Sea-water Injure Egg, Sperm, or Both?

Three explanations of the failure of *Platynereis* eggs to cleave after insemination in sea-water are possible:

(*a*) Both eggs and spermatozoa are injured by the sea-water.

(*b*) The sperm alone are injured by the sea-water.

(*c*) The eggs alone are injured by the sea-water.

The failure of the eggs to go beyond maturation may be due to the injurious action of the sea-water on both eggs and sperm alike. It would seem reasonable to assume that for internal insemination both cells need the perivisceral fluids. It might be difficult to conceive how this adaptation in *Platynereis* could have taken place acting on one only of the sex elements. As both eggs and spermatozoa are protected by body fluids in normal insemination, so both are exposed to the lethal action of sea-water. Embryologists are all careful when inseminating eggs of forms in which insemination normally taken place in the sea not to contaminate the dishes containing ova with the animal's tissues or fluids. Lillie ('13*b*, '14) has shown why this is essential. I have, however, repeatedly with success fertilized *Nereis* eggs dry (see Just, '15*b*) doubtless because the body fluid of *Nereis* is practically negligible. And the case of *Platynereis* is similar to that of *Nereis*; in this smaller worm there is no more fluid; the female is a mere locomotor ovary, although the male does have a small amount of fluid and a great number of corpuscles.

The second possibility is that the sperm alone are injured by the sea-water. Injury to the sperm through transference from the male's body fluid to sea-water, however, cannot be due to difference in osmotic pressure. For as Frédericq has shown, and Garrey since for the Woods Hole region, the osmotic pressure of invertebrate body fluids is about the same as that of sea-water. Moreover, *Platynereis* sperm in sea-water as far as I could determine exhibit none of the effects experimentally produced by Koltzoff on various sperm cells including those of *Nereis* (*dumerilii?*) through treatment by various salt solutions or those conditions described by de Meyer with hypotonic and hypertonic solutions. In some other way, then, the sperm must be assumed to be weakened but still capable of partially fertilizing the egg as the Hertwigs, Gemmil, Budington, Dungay, etc., have shown. And indeed my *Platynereis* slides of sea-water inseminated eggs show similarities to the figures by Lillie of the penetration of injured sperm in *Nereis*; in *Platynereis*, however, the germ nuclei develop

a little farther. Steinach long ago, later Walker ('99, '11) and Hirowaki have shown that in mammals the prostate secretion is necessary for fertilization. Sea-water, then, might injure the sperm and hinder fertilization by destroying a supporting medium necessary for fertilization. (On this point, cf. Gemmil's experiments.)

Finally, a third explanation is possible: the egg alone is injured through sea-water treatment. The egg, in this case, may be dependent on a substance in the female's body or on some secretion of its own necessary for fertilization. Both egg and sperm may need body fluids but sperm may be hardier, egg less resistant.[1]

The seasons of 1912 and 1913 were largely given over to experiments to determine which possible explanation is valid for *Platynereis*. In 1914, many of these experiments were repeated. And I may say at once that the explanation must come under the third head as shown by the following experiments.

The Experiments.

The plan of the experiments is briefly as following:

Males and females were cut up separately in dishes of clean sea-water. The bits of tissue were carefully removed, the dish of eggs being handled with utmost care to prevent unnecessary agitation. The eggs and sperm suspensions were filtered after having remained in sea-water for varying lengths of time. Sexual products treated thus are designated "washed eggs" and "washed sperm."[2]

Males and females were thoroughly dried on filter paper or clean sheer linen. The males were cut up in dried clean watch glasses; the females were cut up in the same way or pricked when

[1] That the resistance of eggs and sperm of both *Nereis* and *Platynereis* is unequal would seem probable from the following: If to a *Nereis* sperm suspension janus green be added the fertilizing power of the sperm is in no wise impaired; or if the dye be added to sea-water the living males absorb it readily without any injurious effect on the sperm. The same quantities of the dye in sea-water is toxic to the egg before or at insemination. Eggs taken from a female *Platynereis* that has been swimming in a janus green-sea-water solution that is not toxic to the males or their sperm will not fertilize. Cf. also action of nicotine on *Strongylocentrotus* sperm and eggs as observed by the Hertwigs.

[2] Several methods were used for "washing" sperm and freeing them of sea-water, among others that of centrifuging at high speed for six minutes. These were all abandoned for the method here described.

most of the eggs that escaped were collected in dry watch crystals. Bits of tissue were always removed. Such eggs and sperm are "dry eggs" and "dry sperm."

For a given experiment eggs and sperm were mixed and after an interval of time varying from five to sixty seconds flooded with sea-water. Four kinds of inseminations were made:

Washed eggs × *washed sperm.*
Washed eggs × *dry sperm.*
Dry eggs × *dry sperm.*
Dry eggs × *washed sperm.*

The experiments fall into two groups: "A.M. inseminations" —made the morning after the worms were captured; and "P.M. inseminations"—made during the evening of capture.

The following table gives a summary of results:

TABLE I.

Eggs.	Sperm.	Group.	Development.
Washed	Washed	A.M. and P.M.	None.
Washed	Dry	A.M. and P.M.	None.
Dry	Dry	A.M. and P.M.	Cleavage and larvæ.
Dry	Washed	A.M.	None.
Dry	Washed	P.M.	Cleavage and larvæ.

Washed eggs, inseminated with dry or washed sperm, never reach cleavage stages nor do they ever produce swimming forms.

I have commented above on the *dry egg* × *dry sperm* series. These eggs cleave and later produce normal larvae.

Washed sperm × *dry eggs* of the A.M. group (1912) did not yield cleavage or swimming forms. The worms do not thrive well in the laboratory. The practise, therefore, of conducting experiments the morning after capture has been since 1912 practically abandoned. The only test for the vitality of the worms is copulation—a test the very nature of which precludes experiment. Doubtless, therefore, this set of experiments gave no results because the animals were not fit. Study of sections of eggs normally inseminated and laid as early as 5 A.M. shows a large percentage in the germinal vesicle stage. I have made counts in dishes of living eggs to show at the later cleavage stages the proportion of eggs still in the germinal vesicle stage. For example,

August 8, 1912, 2 P.M., six hours after laying of 10,851 eggs (from one female) six per cent. were still in the germinal vesicle stage. Other counts of living eggs and of sections show higher percentages. Every egg laid the night of capture cleaves. Dry inseminations, day or night, at best never give more than ninety per cent. of cleavages. The poor quality of the animals after several hours in the laboratory may account for the failure of the *dry eggs* × *washed sperm* A.M. group to cleave. But since the *dry eggs* × *dry sperm* A.M. series gives cleavage, I am rather inclined to believe that the method used was poor: for instance, the filter paper then used was too soft allowing the loss of most of the spermatozoa or too much water was left when the dry eggs were added.

The results with *dry eggs* × *washed sperm*, P.M. group are wonderfully uniform and show conclusively that the sea-water, at least for the exposures used, has no harmful effect on the sperm. The method used is simple. As soon as possible after capture one to three males are cut up in from 8 drops to 20 c.c. of sea-water and allowed to stand upward to twenty minutes. (The sperm are active after having been in sea-water for twelve hours.) The sperm suspension is then filtered. I used a very hard filter paper. This paper was then tilted and thoroughly drained until under the lamplight the glistening water was thoroughly absorbed. A dried female was cut up on the filter paper or pricked and the eggs thus procured rolled over the paper to reach the sperm left behind or caught in the pores of the filter. The whole was then put in a dish of clean sea-water. It would be tedious to cite the individual experiments. They show conclusively that dry eggs inseminated with washed sperm develop in normal fashion.

Now since, as has been shown above, there is a minimal amount of sea-water that will permit fertilization, dry eggs ought to fertilize if put on the filter paper before all the water has been absorbed. Such indeed is the case. Moreover, dry eggs put in two drops of thin sperm suspension develop. From a suspension made by cutting up one or more males in sea-water two drops are taken. Dry eggs put in this cleave and next morning swim.

This observation led to a series of experiments (during 1913 and 1914) designed to ascertain whether or not the density of the sperm suspension is a factor in the fertilization of *Platynereis*.

These experiments prove in general that the number of dry eggs added to sperm suspensions that develop depends upon the density of the suspension. The denser the suspension the larger the number of trochophores. Moreover, for dense suspensions the minimum amount of sea-water permitting fertilization appears to be slightly higher than for thin suspensions. Cleavage is directly a function of the chances of the spermatozoa reaching the egg before the fertilizing substance is lost.

The time of flooding with sea-water after insemination is also important for the highest percentage of cleavage. But these factors cannot be expressed with mathematical exactness. Some points, particularly with reference to inseminations with dense suspension need further experiments to determine their significance.

That the egg when exposed to the action of sea-water quickly loses something necessary for fertilization must be the conclusion drawn from these experiments with washed or unwashed eggs. Even *thirty seconds* residence in sea-water, as repeatedly proved, is sufficient to inhibit cleavage in every single egg. If dry eggs from a single female be put in five cubic centimeters of sea-water and thoroughly drained as soon as they settle they will not develop after insemination although this procedure may take but a half minute. The egg alone is affected by sea-water; the fertilizing power of the sperm is not affected by exposure to sea-water.

3. *The Nature of the Fertilizing Substance.*

The fertilizing substance once lost cannot be restored. If washed eggs be mixed with an extract obtained by crushing dry eggs in one or two drops of sea-water and dry sperm added, cleavage does not result. I lay no stress on this, however, for it seems to me that such an extract might yield anything.

The presence of various substances in the sea-water or the lowering of the temperature of the sea-water does not prevent or restore the loss of this substance.

KOH.—Eggs were teased out of the female directly into sea-water plus KOH in various proportions. Or, eggs from dried females were placed in the solution. After remaining from thirty seconds to two minutes in the alkaline sea-water the eggs were inseminated dry and flooded with sea-water. In other cases inseminations were made in the solutions. Washed eggs were similarly treated. Whatever the method alkaline sea-water never gave cleavage. (Cf. sections on cross fertilization and artificial parthenogenesis.)

Hypertonic and Hypotonic Sea-water.—Eggs, both washed and dry, were treated with 2½ M KCl + sea-water as follows:

1. 1 drop 2½ M KCl + 19 drops of sea-water.
2. 2 drops " " + 18 " " "
3. 3 " " " + 17 " " "
4. 4 " " " + 16 " " "
5. 5 " " " + 15 " " "
6. 6 " " " + 14 " " "

Dry sperm were added at once and the dishes flooded with sea-water after five minutes. Or, after treatment for varying number of minutes the eggs were inseminated dry. The eggs developed no farther than with KCl treatment alone (see beyond); they form jelly and maturate.

Hypotonic solutions used similarly gave no cleavage.

Ether.—The following table is a summary of the experiments with ether:

Eggs.	Solutions Used.	Exposure.	Inseminations.
Washed,	.3 to .6 per cent.	1 to 5 minutes dry;	in the solution.
Dry,	"	" "	" " "
Teased,	"	" "	" " "

"Teased" eggs are those got by cutting up the female in the ether-sea-water.

A few eggs form jelly and maturate after the ether treatment. Compared with sea-water inseminations, ether cuts down the per cent. of maturations. According to R. S. Lillie ('12) starfish eggs resistant to fertilization may be rendered normal by ether in low concentration. In *Platynereis* the condition is different. The egg is not rendered resistant to fertilization by the action of sea-water; it is weakened through loss of something

by the sea-water since it combines but feebly with the sperm The ether as in *Asterias* renders the *Platynereis* egg irritable since as shown by the low percentage of maturation more fertilizing substance must be secreted.

KCN.—Inseminations made with washed or dry eggs during or after treatment with KCN (1 per cent. KCN and sea-water made in various proportions) gave only maturation. But the eggs will maturate in KCN alone while in the solutions. (Cf. Allyn on *Chætopterus.*)

$CaCl_2$.—Newman found that $CaCl_2$ inhibits fertilization in *Fundulus* through a precipitation effect. I thought that in somewhat the same way calcium chloride might through action on the cortex inhibit the loss of the fertilizing substance in *Platynereis.* M/2 $CaCl_2$ added to sea-water in different quantities does not inhibit the loss of the substance since after the calcium chloride treatment the egg does not fertilize.

Cooled Sea-water.—Sea-water was cooled to 10.5° C. and dry eggs after 30, 60 and 90 seconds' treatment in 5 c.c. were inseminated at this temperature or after the cooled water was pipetted off. In some experiments the female was kept at the low temperature for several minutes before the eggs were cut out. 5 c.c. of sea-water were used in each experiment. The eggs never cleave, but more form jelly and maturate than controls inseminated in ordinary sea-water. This would seem to indicate a slowing down of the secretion. The effect of cold is just the opposite of the effect of ether. Unfortunately, only few of these experiments were made. Perhaps they should be repeated at lower temperatures.

Concerning the nature of this substance, some of my earliest notes are of interest. After insemination in sea-water I found some time later (forty minutes in one case) "sperm dancing above the eggs." In 1914, I found the sperm of sea-water insemination active after twelve hours. One does not find this after dry insemination, even with excess of sperm. Sperm in the dishes of successfully inseminated eggs are profoundly changed. Study of the movements of *Platynereis* sperm reveals the circular swimming of echinid spermatozoa, as shown by Buller, Gemmil, Winslow, and others (see also Dewitz, Ballowitz, etc.). They

finally become quiescent through lack of oxygen[1] in various positions without orientation. After dry inseminations they come to rest, as can be seen after flooding the dishes, definitely oriented and not in haphazard arrangement. Clustered among the jelly hulls, their heads point toward the eggs. On occasions, I believed that I demonstrated the agglutination of the sperm by sea-water in which the eggs had been lying. The evidence is not clear-cut and more recent attempts have failed. The egg charged sea-water, however, does activate the sperm.

I wish to point out the serious difficulties experienced in the series of sperm agglutination experiments. In the first place, twenty "large" dried males (two and one half centimeters long) do not yield enough sperm and body fluid to make up a drop as large as a drop of dry sperm from a very small *Nereis*. Then again the thickest suspension got is largely made up of blood corpuscles. I have never succeeded in procuring a "milky suspension"—the admixture of corpuscles and body fluid giving always a pinkish mixture. And finally, one cannot always get twenty or more males necessary to make up even this thin sperm suspension. Repeated efforts, therefore, extending through two seasons have not been marked with very positive results.

With *Nereis* sperm, the case is indisputable. If water in which *Platynereis* have laid eggs be taken it is found to have an agglutinating effect on *Nereis* sperm. Thus:

August 18, 1914. At 10:15 P.M., ten females laid eggs in six c.c. of sea-water each. After five minutes some of this water was drawn off—20 c.c. in all. *Nereis* sperm suspensions were made up fresh at 10:20, 10:30, 11:00 and 11:05. A drop of the sperm suspension was mounted on a slide under a raised cover slip. A drop of the water taken from the dishes of eggs was injected beneath the cover slip. Under the microscope, the quiescent sperm appeared at first intensely active, then rushed together and formed agglutinated masses among others still free-swimming.

[1] This fact was brought out in 1913 when I was repeating some old observations on echinoderm spermatozoa. While experimenting with the sperm of *Thyone* in janus green solutions, I noted after some time had elapsed that cover-slip preparations showed that bacteria present previously bluish in color had changed to a decided red. Later observations proved that as the dye was reduced in bits of tissue under the cover slip the sperm quieted down in various positions.

The same experiment succeeds if one uses the water from dishes in which uninseminated eggs have remained for a few minutes. Washed eggs do not cause agglutination of *Nereis* sperm; water charged by normally inseminated eggs or uninseminated eggs retains its power of agglutinating *Nereis* sperm after twelve hours at least, the reaction coming on more slowly. The freshly charged water acting on fresh sperm suspension gives a clear-cut and beautiful reaction.

It may seem far-fetched to argue that the fertilizing substance lost by *Platynereis* eggs when exposed to sea-water is agglutinin or fertilizin as discovered by Lillie in *Nereis* and *Arbacia* because the washed egg, no longer fertilizable by its own sperm, can not sufficiently charge the sea-water to agglutinate *Nereis* sperm. Yet I believe this is the case precisely. The agglutination of *Nereis* sperm by *Platynereis* egg-water is correlated with jelly formation in *Platynereis* by *Nereis* sperm. In sea-water inseminations, *Nereis* spermatozoa are almost as effective as those of *Platynereis*. Added to this is the difference in behavior of *Platynereis* sperm in egg charged sea-water, in sea-water inseminations, and in dry inseminations.

The evidence may be scant, but it seems to me sufficient to indicate that the substance lost which is necessary for fertilization is identical in nature with the fertilizin of Lillie.

B. Cross Fertilization with Nereis.

I have mentioned (Just, '14) the fact that it is generally taken for granted that reciprocal crossing of *Nereis* and *Platynereis* is the rule. This led me to attempt cross fertilization. Cross fertilization never produces segmentation or development though it may induce the maturation process.

Of the methods used in echinoderm hybridization—those of Loeb, Tennent,[1] etc.: (1) high temperature; (2) treatment with fresh water; (3) treatment with alkalis; (4) allowing the eggs to stand; and (5) polyspermy—all were tried except the first. Since the eggs of *Platynereis* are normally inseminated in the body cavity and therefore with little sea-water, I tried "dry

[1] Dr. Tennent in 1912 very kindly communicated to me at length his latest methods in echinoderm hybridization.

inseminations": *i. e.*, *Nereis* males were cut up dry and a drop of the sperm without the addition of sea-water added to eggs of *Platynereis* cut up dry. Inseminations were made in a variety of ways as the following table of method shows:

TABLE II.

SUMMARY OF INSEMINATIONS MADE IN 1911, 1912, 1913, AND 1914

Platynereis sperm	on	*Nereis* egg.
1. Few sperm in sea-water. 2. Dense sperm suspension.		Fresh eggs in sea-water.
3. Few sperm in sea-water. 4. Dense sperm suspension.		Stale eggs in sea-water.
5. Few sperm, dry, 6. Heavy insemination dry.		Fresh eggs dry.
7. Few sperm, dry. 8. Heavy insemination, dry.		Stale eggs washed.

Reciprocal crosses of *Platynereis* eggs and *Nereis* sperm were made.

"Stale eggs" are eggs that have stood in sea-water for several hours. "Stale eggs, washed" are stale eggs on which the water has been changed several times.

These experiments were made repeatedly during four seasons. The sperm of *Platynereis* has practically no effect on the egg of *Nereis* whether fresh or stale, dry or in sea-water. In one experiment (1911) I got jelly formation in a few eggs. This experiment later repeated (1913) gave no result. If *Nereis* eggs be inseminated with *Platynereis* sperm during the evening of capture they show no change the next morning. Inseminated with *Nereis* sperm twelve hours after insemination with *Platynereis* sperm, the eggs develop normally if anything in greater numbers than such stale eggs in ordinary sea-water do.

Nereis sperm will cause *Platynereis* eggs to form jelly, the per cent. of eggs thus responding depending upon the amount of sea-water used and the density of the sperm suspension. But in general many of the eggs fail to form jelly or go through maturation. Many that maturate do so with the cortex partially or wholly intact. Sections of these eggs preserved at three minute

intervals after insemination have been studied. The sperm does not enter; or, if it enters must disintegrate early for I have never found sperm nuclei in these preparations.

Clearly, then, one may not use the eggs of these worms indiscriminately.

C. Artificial Parthenogenesis.

The following agents have been used in an attempt to bring about artificial parthenogenesis in the egg of *Platynereis megalops*:

1. Centrifuging,
2. KCl,
3. NaOH,
4. KOH,
5. HNO_3,
6. HCl,
7. Warm sea-water.

The eggs were cut out of the worms in sea-water centrifuged; subjected to varying quantities of salt, alkalis, or acids for different lengths of time; or warmed in sea-water for from five to thirty minutes at 35° C. These methods gave polar body formation, cytoplasmic changes, fusion of the oil drops, and finally chromatin disintegration in the animal hemisphere. The eggs never cleaved.

Study of the literature reveals the fact that the clearest cases of artificial parthenogenesis closely simulating the normal in cleavage and in larval development are of those eggs that have formed one or both polar bodies when shed: the echinids, for example, and the asteroids. Other eggs shed in the germinal vesicle stage like those of *Polynoe* (Loeb '08), *Amphitrite* (Loeb '01; Scott.) *Nereis* (Lillie '11), etc., give only differentiation without cleavage or incomplete cleavage. Loeb and Wasteneys' work on *Chaetopterus* with ox serum as well as Miss Allyn's on the same egg with heat are exceptions. The great exception to the general statement made above is *Thalasema* (Lefevre) where it appears with single substances, acids mostly, normal development is closely simulated. On the whole, however, ovocytes yield less readily to parthenogenetic agents than mature ova.

Mathews' experiments ('01) on *Asterias* may in this connection be cited. He found that when the eggs of this starfish were got while still in the germinal vesicle stage shaking would produce development only after the eggs had remained in sea-water until maturation was gone through with. Sea-water acts as a first stimulus and mechanical shock induces further development. So R. S. Lillie ('08) on the same egg finds that its responsiveness to momentary elevation of temperature as a means of producing artificial parthenogenesis "varies greatly at different periods in the life of the egg." "The most favorable period is some little time (10 to 20 minutes) before the separation of the first polar body."

Reasoning thus, I thought that I might carry *Platynereis* eggs through maturation with one agent and then through cleavage with another. Eggs were, therefore, treated with KCl, KOH, and NaOH in sea-water for various lengths of time and then subjected to heat, shaking, and centrifugal force. In no case did I procure cleavage although the first agent in each case caused maturation. With *Nereis*, on the other hand, KCl and subsequent warming in sea-water induces development (see Just '15*b*).

It is interesting to note that eggs subjected to heat in the minute quantities of sea-water that permit fertilization do not develop beyond maturation. Apparently, the conditions for successful artificial initiation of development are more exacting than those for successful insemination.

We may conclude, then, that the results of attempted cross fertilization and artificial parthenogenesis are harmonious with those of sea-water insemination, so far as cleavage is concerned, in their negative results. The fundamental questions are: (1) the significance of the sea-water insemination and (2) the extent to which the results with *Nereis* sperm and with parthenogenetic agents are capable of like interpretation.

Discussion.

Any analysis of fertilization must deal with the phenomena from the point of view of heredity or of initiation of development. Considered as the process of initiating development, fertilization may be divided into the stages of insemination, sperm penetration, and germ nuclei copulation. As Lillie has repeatedly

pointed out[1] experimental evidence must be amassed testing the meaning of each of these stages.

1. Concerning insemination, as Lillie has shown, the egg plays an important part through the production of agglutinins.[2] For both *Arbacia* and *Nereis* it has also been shown that chemotaxis plays a part in insemination. (Lillie, '12, '13*a*, '13*b*, and '14).

I believe that *Platynereis* belongs to this class. I may, however, be permitted again to point out the great difficulty attending the use of *Platynereis* eggs on this phase. All the phenomena are extremely rapid, the reactions must be very nice. The material is unfavorable for any intensive study of agglutination and chemotaxis. When one stops to think of the extremely precise reactions of the eggs, one gets a hint of the task. The carrying over of the *smallest* drop of sea-water above the maximum to eggs from vigorous females within the shortest time after capture will prohibit cleavage in every egg.

To answer the general question whether or not eggs secrete substances that activate the spermatozoa, I believe forms whose eggs are inseminated normally in sea-water should be used. So far as *Platynereis* is concerned, agglutination or not, chemotaxis or not, the egg must lose a substance or substances when in sea-water whose presence is necessary for fertilization.

2. Study of the normal fertilization of *Platynereis* indicates that as in *Nereis* the egg plays the active rôle in the penetration of the spermatozoön for it actually draws in the passive spermatozoön. After sea-water treatment I have not, as mentioned above, found the early stages of penetration in eggs fixed at three minute intervals after insemination. Either the sperm penetration is unlike that after normal insemination or penetration takes place with extreme rapidity. In the later stages of penetration it is

[1] Lectures to classes in embryology, Woods Hole, Mass.

[2] Apparently Buller did not realize that he obtained iso-agglutination of sea-urchin sperm, although he speaks of the sperm forming "balls" and although the phenomena of agglutination were well known at that time. Landsteiner the year before had secured sperm agglutinating sera. Nougouchi's work on *Nereis* sperm is of interest: he demonstrated agglutination with snake venom. The experiments of Schücking, von Dungern, de Meyer, and others are well known. An observation of Walker's ('10) is likewise worthy of mention—the agglutination of the sperm of the rat when mixed with the seminal vesicle secretion of the same animal.

Chemotaxis of sperm has been demonstrated for mammals—see for instance, Löw.

clear that the spermatozoa behave in abnormal fashion even granting that I may have overlooked the amphiaster. The evidence seems to indicate that after sea-water treatment the egg lacks the power to engulf the sperm. However, whatever the method of penetration one point is beyond contradiction: these washed eggs never cleave.

The observations agree with those of Lillie ('14) who notes that some unpublished observations in the case of *Nereis* show that "if the cortical changes be induced by artificial means there is a brief period in which insemination of the eggs may be followed by penetration of the spermatozoön, but without causing cleavage of the egg." Miss Allyn found that after KCl treatment of the egg of *Chætopterus*, the spermatozoön may enter but its behavior is not normal. Kite (quoted from Lillie '14) finds that spermatozoa injected into star-fish eggs never give cleavage.

In these cases, the interpretation must be that the "fertilizable" condition of the egg has been destroyed through loss of fertilizin before insemination. In the same way sperm may penetrate unripe eggs as Hempelmann has shown for *Saccocirrus* (so too, von Hofsten for *Otomesostoma* and Shearer for *Dinophilus gyrociliatus*). Two years ago I found that eggs from *Nereis limbata* just before transformation into the heteronereis phase would not fertilize with active sperm either from the nereis or heteroneris form. Moreover, eggs from metamorphosing worms kept for several weeks in the laboratory although apparently ripe would not fertilize on insemination during the dark of the moon. At full moon, sometimes but a few days later, eggs from the same animal would fertilize and develop into larvæ which were kept for weeks. We may assume in these cases that the fertilizin is either absent or is unavailable. Penetration, therefore, may take place before the fertilizable period is reached as well as after it has been passed, but the egg is not capable of fertilization.

3. Apposition of the germ nuclei of *Platynereis* after sea-water insemination may ensue, but never cleavage. After the loss of the fertilizing substance, then, the normal fertilization process may be closely simulated even to the point of the copulation of the pronuclei but development never goes beyond this point. In short, the normal fertilization process demands at the very

outset the fixation by the spermatozoön of the escaping fertilizin. This takes place in *Platynereis* almost instantaneously (see page 93) but brief though this phase may be it cannot be omitted.

The experiments with *Nereis* sperm and agents of artificial parthenogenesis demand explanation. Eggs such as those of echinids used in cross fertilization (Loeb, Tennent, Baltzer, Herbst, etc.) or in artificial parthenogenesis when subjected to treatment are so subjected with their substances intact. They are normally shed in sea-water for insemination and the sea-water does not for some time destroy their fertilizing power. *Platynereis* eggs when subjected in sea-water to foreign sperm or to various agents have lost something through the action of sea-water. This very "something" is necessary for artificial parthenogenesis and, moreover, as shown above (for *Nereis* also) must be present in greater quantity than necessary for fertilization. I am emboldened further to suggest that eggs normally inseminated in the ovocyte stage yield to parthenogenetic agents only with difficulty because they lose fertilizin at the impact of the first stimulus—chemical treatment, shock, etc. Sperm alone, in most cases, are strong enough by fixation of the fertilizin to carry such eggs through their dual phase—maturation and fertilization. Whether by sperm, then, or by artificial agents, the initiation of development is fundamentally the same.[1] The egg plays the leading rôle; it needs but to have its fertilizin activated in order to develop.

The observations on *Platynereis* were rendered less difficult because of the study of the maturation and fertilization in *Nereis*. For this study I was fortunate to be able to supplement my own slides with two series lent me by Professor F. R. Lillie. It is a genuine pleasure here to acknowledge my further indebtedness to him for his many suggestions and for his stimulating interest in the *Platynereis* studies begun at his suggestion and under his direction.

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[1] I think that Martin Jacoby's experiments support this view. He found (*Biochem. Zeit., 26, 333–335*) that serum from rabbits into which eggs had been injected showed an increased power to stimulate parthenogenetic development of the eggs. He also found (*ibid., pp. 336–343*) that an enzyme which may be extracted from sperm and from eggs after sperm penetration may be got from parthenogenetic eggs.

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